# REDUCTION OF ALKYL HYDROPEROXIDES TO ALCOHOLS: ROLE OF RUBREDOXIN, AN ELECTRON CARRIER IN THE BACTERIAL HYDROXYLATION OF HYDROCARBONS*

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Summary: Alkyl hydroperoxides are reduced to alcohols in the presence of DPNH and homogeneous rubredoxin and rubredoxin-DPN reductase from Pseudomonas oleovorans. The reaction does not occur when TPNH is substituted for DPNH or when rubredoxins from anaerobic bacteria are substituted for the P. oleovorans nonheme iron protein. The reaction appears to have broad substrate specificity; 1-octyl, 2-octyl, cyclohexyl, and cumyl hydroperoxides are all reduced at significant rates. The reduction of alkyl hydroperoxides is inhibited by cyanide, as is the hydroxylation of alkanes and fatty acids in the same enzyme system supplemented with the ω-hydroxylase.

Previous studies in this laboratory have shown that alkanes (1,2) and fatty acids (3,4) undergo hydroxylation in enzyme preparations of Pseudomonas oleovorans according to the equation: $RCH_3 + DPNH + H^+ + O_2 \rightarrow RCH_2OH + DPN^+ + H_2O$. The three enzymes found to be required in the overall reaction (5) were purified and identified as rubredoxin (6,7) rubredoxin-DPN reductase (8), and an ω-hydroxylase (9). In the present paper evidence is presented that rubredoxin serves as an electron carrier in the reduction of alkyl hydroperoxides to the corresponding alcohols.

Hydroperoxides were apparently first suggested as intermediates in microbial hydrocarbon oxidations by Imelik (10) in 1948, and numerous investigators have subsequently proposed a role for hydroperoxides or have studied their fate

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in biological systems, as reviewed by McKenna and Kallio (11). Alkyl hydroperoxides are known to be metabolized by bacterial cells (12-14), and fatty acid hydroperoxides are known to be decomposed by transition metal ions or heme compounds, as well as by subcellular fractions from rat liver (15,16), or by liver or erythrocyte glutathione peroxidase (17,18). Chen and Lin (19,20) have recently reported that hydroperoxides are intermediates in the hydroxylation of tetralin and fluorene in a rat liver supernatant fraction. The studies described in the present paper apparently provide the first example of hydroperoxide reduction by a nonheme iron protein or, for that matter, by any highly purified enzyme known to play a role in biological hydroxylation.

In the course of recent studies on the mechanism of octane hydroxylation, various possible intermediates were synthesized and tested for their ability to yield the corresponding alcohol when added to the P. oleovorans enzyme system.

Table I

Components required for reduction of alkyl hydroperoxide

| System | % Maximal activity |
|---|---|
| Complete | 100 |
| No octyl hydroperoxide | 2 |
| No DPNH | 0 |
| TPNH substituted for DPNH | 4 |
| No rubredoxin | 1 |
| No reductase | 5 |
| Complete + 5 x $10^{-3}$M KCN | 35 |
| Complete + $10^{-3}$M EDTA | 15 |
| Complete, but with boiled rubredoxin | 13 |

The complete reaction mixture contained, in a final volume of 1.0 ml, 100 µmoles of Tris buffer, pH 7.4, 0.2 µmole of DPNH (or TPNH), 0.27 mg of (1Fe)-rubredoxin (7), purified rubredoxin-DPN reductase (1.5 µg of protein), and 0.5 µmole of 1-octyl hydroperoxide (in 0.01 ml of acetone) as the final addition. The rate at 30° in the complete system, measured as $-\Delta A_{340}$ per min, was 0.196. The alkyl hydroperoxides used in this study were prepared by a published procedure (21) and the purity was established by thin layer chromatography and infrared spectroscopy; gas liquid chromatography yielded octaldehyde as the major decomposition product.

1-Octyl hydroperoxide proved to be of particular interest because it caused rapid oxidation of DPNH, as shown in Table 1. No significant reaction was observed when TPNH was substituted for DPNH or when rubredoxin or the reductase was omitted. The ω-hydroxylase was not present in these experiments and, in fact, was found to be somewhat inhibitory when added. As also shown in the table, cyanide and EDTA cause extensive inhibition when added to the complete system. The product was identified as n-octanol by thin layer and gas liquid chromatography. Radioactive 1-octyl hydroperoxide was used to establish that product formation is equimolar with respect to DPNH disappearance, as shown in Table II.

Table II

Stoichiometry of hydroperoxide reduction

| Expt. | Conditions | DPNH oxidized (nmoles) | Alcohol formed (nmoles) | DPNH / Alcohol (molar ratio) |
|---|---|---|---|---|
| 1 | Complete system | 70 | 66 | 1.06 |
| 2 | Complete system + carrier n-octanol (0.15 μmole) | 75 | 69 | 1.09 |
| 3 | Rubredoxin omitted | 0 | 0 | |

1-$^{14}$C-n-Octyl hydroperoxide (0.15 μmole; 4.5 x $10^3$ cpm) was used as substrate in the usual reaction mixture. DPNH disappearance was estimated spectrophotometrically; at 20 min the reaction mixture was extracted with ether and the extract was concentrated and submitted to thin layer chromatography in 20:1 benzene-ethyl ether. The $^{14}$C-labeled octanol ($R_f$ 0.14), which was well separated from the hydroperoxide ($R_f$ 0.32), was removed and the radioactivity was determined in a scintillation counter.

The reduction of octyl hydroperoxide is linear with respect to the rubredoxin concentration over the range shown in Fig. 1. On the other hand, rubredoxins from anaerobes such as Peptostreptococcus elsdenii, Clostridium pasteurianum, and Desulfovibrio gigas (all of which are rapidly reduced by the

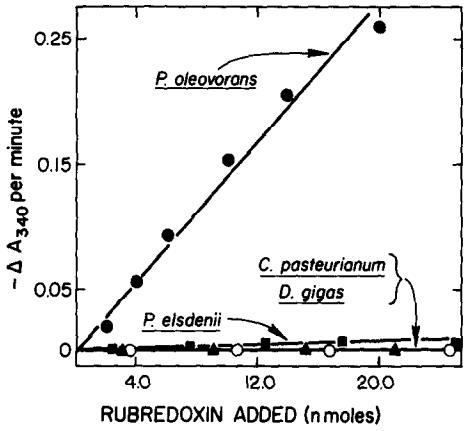


Fig. 1. Octyl hydroperoxide-dependent DPNH disappearance as a function of rubredoxin concentration. The conditions were as in Table I, except that a higher level of reductase was used (3.5 µg), and various rubredoxins were at the levels indicated.

bacterial reductase) had no significant activity even at high concentrations. The substrate specificity appears to be broad, however. As shown in Fig. 2, besides 1-octyl hydroperoxide, 2-octyl and cyclohexyl hydroperoxides are also effective substrates; the corresponding alcohols were identified as the products. The apparent Km values are 2 x $10^{-4}$, 1 x $10^{-4}$, and 1 x $10^{-4}$M, respectively. It may be noted that, in these experiments, TPNH and spinach ferredoxin-TPN re-

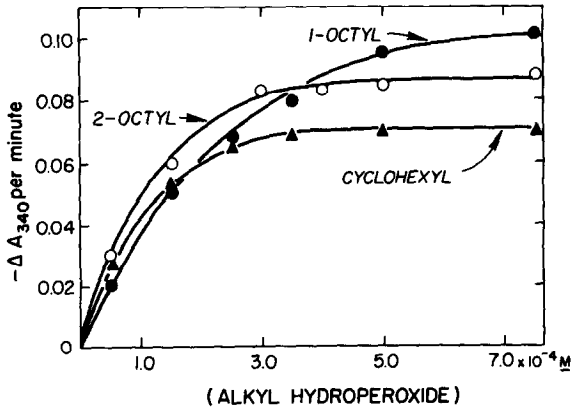


Fig. 2. TPNH oxidation as a function of alkyl hydroperoxide concentration. The conditions were as in Table I, except that TPNH was substituted for DPNH and spinach ferredoxin-TPN reductase (20 µg) for the bacterial reductase. Acetone, which had no effect on the reaction, was at a final concentration of 1.4% at the highest alkyl hydroperoxide levels.

ductase were substituted for DPNH and the bacterial reductase. In other experiments cumyl hydroperoxide, hydrogen peroxide, and benzoyl peroxide were also shown to cause the oxidation of reduced pyridine nucleotide in the presence of rubredoxin and the reductase. Octaldehyde, a known thermal decomposition product of 1-octyl hydroperoxide, showed no significant activity in the system. The reaction may, therefore, be written as follows:

$$ROOH + DPNH + H^+ \longrightarrow ROH + H_2O + DPN^+,$$

where R may be a primary, secondary or tertiary alkyl group.

These results clearly establish that P. oleovorans rubredoxin serves as an electron carrier for hydroperoxide reduction, but the significance of the reaction is not yet clear. Attempts to identify an alkyl hydroperoxide as a free intermediate in the hydroxylation of alkanes have so far proved negative. On the other hand, an enzyme-bound hydroperoxide could not have been detected. Some similarities in hydroperoxide reduction and in substrate hydroxylation in the P. oleovorans enzyme system should be emphasized. Both reactions are specific for rubredoxin from P. oleovorans, have the same pH optimum (7.4), and are inhibited to a similar extent by cyanide. Furthermore, the rates of the two reactions appear to be similar when rubredoxin is the limiting component. It should be noted that the reaction of an alkane with $O_2$ to form the hydroperoxide, followed by the reduction reaction described in the present paper, would constitute a hydroxylation reaction having the expected overall stoichiometry. The possibility may also be considered that the function of rubredoxin in aerobic cells is to decompose hydroperoxides by a reaction unrelated to substrate hydroxylation.

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